Effects of Breast Cancer Cell-Derived Extracellular Vesicles on Endothelial Cells and Angiogenesis

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Abstract - The severity of breast cancer is greatly increased once cancer cells undergo metastasis and spread throughout the body. Interactions between our body's endothelial cells (EC) and extracellular vesicles (EV) excreted from breast cancer cells are thought to induce metastasis— endothelial cells are responsible for angiogenesis, the formation of new blood vessels, and create new pathways for cancer cells to spread. In this investigation, the effects of breast cancer cell derived EV on EC are observed through changes in the angiogenic ability of EC. Angiogenic ability before and after successful EV to EC incorporation was observed in in vitro environments. Results displayed that EV influenced EC activity to enhance cancer metastasis. With added EV, EC angiogenesis ability intensified to form larger networks of tubes formation in in vitro environments. Therefore, breast cancer cell EV play a role in the escalation of cancer metastasis via angiogenesis and EC — the prevention or restriction of breast cancer would lie heavily in further understanding of EV.

Key Words - Extracellular vesicles (EV), endothelial cells (EC), angiogenesis, metastasis, tube formation

INTRODUCTION

I. Research Question

What effects do breast cancer cell-derived extracellular vesicle have on human endothelial cells and, consequently, angiogenesis?

II. Background Context

Breast cancer is the accumulation of abnormal, cancerous cells in the breast area and is the most common. cancer for women worldwide [1]. 1 in 8 women in the United States will develop breast cancer at least once in her lifetime and in 2018 alone, approximately 2.1 million new cases of breast cancer were diagnosed [2]. The survival rate of breast cancer patients is heavily dependent on the level of metastasis, the spread of cancer cells to different parts of the body [3].

Metastasis can occur when cancerous tumor cells disseminate either through the blood vessels or the lymphatic vessels. Angiogenesis, the creation of new blood vessels, therefore plays a significant role in metastasis and tumor growth in different parts of the body as angiogenesis creates new pathways for cancer to spread. This investigation delves into the effects that breast cancer cells (BCC), their DNA dense extracellular vesicles (EV) in particular, have on the body's angiogenic ability and metastasis. These effects are investigated by incorporating BCC EVs with our body's endothelial cells (EC), which are vital in the production of new blood vessels and angiogenesis [5], and observing the effects on of angiogenic ability.

LITERATURE REVIEW

I. Breast Cancer and Metastasis

Metastasis occurs when cancerous cells spread to different parts of the body, away from its origin [6], and is the most important factor in determining the survival rate of the breast cancer patient. When the breast cancer cells have not metastasized, women have a high 5-year survival rate of 99% [7]. However, once breast cancer cells undergo metastasis and spread to various parts of the body, the 5 year survival rate significantly drops to 27% [7]. Metastatic breast cancer cells are difficult to treat by conventional methods such as surgery or radiotherapy since the cancer cells have already spread away from the origin and are much harder to pinpoint and treat [8]. The prevention of breast cancer cells from reaching this stage of metastasis would therefore increase mortality rate of breast cancer patients.

II. *Role of Angiogenesis in Metastasis*

Angiogenesis is a critical process required for metastasis. Angiogenesis is the formation of new blood vessels from pre-existing blood vessels [9] and, under non-cancerous conditions, is a typical body process during one's growth and wound healing. However, in cancerous conditions, angiogenesis is an essential step in the development of new metastatic pathways in which the BCC can enter the bloodstream and spread [10].

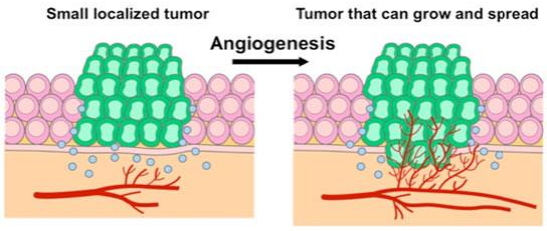

FIGURE 1: Role of Angiogenesis in the Spread of BCC [11]

New blood vessels created via angiogenesis provide new routes by which tumor and cancer cells exit the original tumor site and enter the circulation — triggering metastasis (Fig. 1) [10]. Instead of the tumor simply growing larger in size to reach pre-existing blood vessels, BCC could *possibly* influence angiogenesis to create new blood vessels to reach the tumor itself — this investigation will study the degree to which BCC will be able to influence angiogenesis and therefore, metastasis.

III. Endothelial Cells and Angiogenesis

Endothelial cells (EC) form the single cell layer that lines all blood vessels and the proliferation in EC numbers are accompanied with the rise of mature blood vessels—making EC vital for angiogenesis [12]. EC assist in angiogenesis by forming capillary-like structures, and extending and remodeling pre-existing blood vessels [13]. ECs are nearly always found near the tumor microenvironment [14] and can directly impact the rate of angiogenesis. Therefore, changes in EC activity after BCC incorporation compared to EC activity without incorporation will strongly indicate that BCC will also influence angiogenic ability.

IV. Breast Cancer Cell-Derived Extracellular Vesicles and Endothelial Cells

BCC can influence our body's endothelial cells through interactions between the BCC's extracellular vesicles (EV) and the body's endothelial cells (EC). EVs are membranous structures excreted from cells and often act as a biological cargo for lipids, proteins, and (most importantly) RNAs and DNAs [15]. EVs excreted by BCC allow for intercellular communication between cancerous cells and our body's cells through the exchange of genetic material [16]. Several past studies exemplify how strands of DNA and RNA in the EV could be selectively packaged *and* functional in their target cells [17]. In theory then, incorporating EVs derived from breast cancer cells with endothelial cells should cause an exchange of genetic material between EV and the body's EC.

PURPOSE OF STUDY

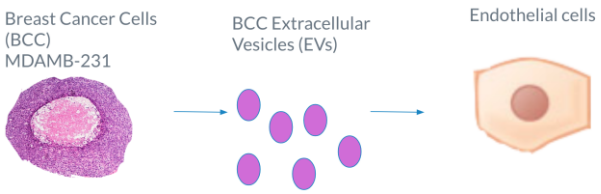

FIGURE 2: Flow Chart Exemplifying Effects of BCC to EC

I. Objective

The objective of this investigation is to examine the effects of BCC on EC through intercellular interactions through the genetic material found in BCC derived EV. As seen in Figure 2, BCC will excrete EV (filled with genetic material) and in turn affect EC and its behaviors in angiogenesis and metastasis. Investigating intercellular interactions between BCC derived EV and EC would deepen our understanding in the role of BCC EV in angiogenesis, which could be applied to clinical procedures in the prevention of metastasis.

II. Phenotype investigated

The phenotype investigated are changes in angiogenic ability. Results will compare differences between the control (only EC) versus the variable (EC with EV incorporated in them). Angiogenic ability is investigated through an endothelial cell tube formation assay. Both the control and the variable are placed in *in vitro* conditions which mimic the body's microenvironment (matrigel and nutrients) to allow the endothelial cells to create tube formations in the gel. These tube formations represent blood vessel formations in the body. Differences between tubes formed by the control and the variable exemplify differences in angiogenic ability due to EV incorporation.

HYPOTHESIS

I. Hypothesis for Angiogenic Ability

After EV incorporation, the EC+EV (EC with added EV) samples should have increased tube formations in the *in vitro* conditions compared to samples only containing EC. Tubes formed by EC+EV samples should be longer in length and form more extensive networks. As these tube formations represent blood vessels, the degree of impact that BCC EVs would have on our body's EC and angiogenesis will be determined.

METHODOLOGY: INCORPORATION OF EV TO EC

I. Justification

This investigation requires the comparison between EC and EC+EV. The EC used is store bought Human Umbilical Vein Endothelial Cells (HUVEC). However, the EV are derived from breast cancer cells straight from the hospital. From the range of different types of BCC, MDA-MB-231 is used. MDA-MB-231 is a triple negative cancer, and metastases easily, making this BCC a suitable sample in this investigation. To obtain only EV from BCC, a thorough separation procedure is needed. The standard method for EV extraction [18] was slightly adjusted for this investigation but the underlying principles stayed the same.

II. Experimental Procedure

BCC solution was obtained directly from the hospital. The BCC solution was poured into 6 micro-centrifuge tubes and centrifuged under 1000rpm, 4°C for 5 minutes. The BCC solution was centrifuged again under 2100rpm, 4°C for 20 minutes. After ensuring the micro-centrifuge tubes were not damaged, the BCC solutions were centrifuged in a super centrifuge under 37000 rpm for 70 minutes. The supernatants were carefully removed from the micro-centrifuge tubes using a mechanical pipette. The EVs found in the precipitate were stained with PKH26. PBS was added to fill up the micro-centrifuge tubes so nearly the entire tube was filled. Again, the PBS and EV mixture was centrifuged in a super centrifuge under 37000rpm for 70 minutes. The PBS and EV mixture was removed and the EV concentration obtained using a spectrophotometer [19]. The EV concentration for this investigation was 0.087 μg/mL.
To create samples, 60mL of HUVEC [20] was added into 10 cell culture dishes. Five of the cell culture dishes were labeled as "EC" while the remaining were labeled as "EC+EV" using tape and marker. 69mL of EV solution was added to the cell culture dishes labels "EC+EV" (*Refer to Calculation: Volume for Assay Samples to find how volume of 69mL was determined)*. The cell culture dishes labeled "EC+EV" were mixed by slowly rotating the dishes. All 10 dishes were left in an incubator at 36°C for 24 hours.

METHODOLOGY: ANGIOGENIC ASSAY

I. Justification

To observe the formation of new blood vessels, the samples will be placed in *in vitro* conditions to mimic the body's microenvironment. The tube formation assay is a widely used *in vitro* assay which model the ability of EC to perform angiogenesis [21], and will be used in this investigation. The samples placed *in vitro* will form tubes which represent blood vessel formation in the body. The sample cells will be placed in Matrigel Growth Factor Reduced, a gel used to commonly used to culture cells. The preparation of the matrigel and the tube formation assay was followed accordingly to the procedure provided [22] with adjustments to include the sample of EC+EV.

II. Experimental Procedure

2mL of matrigel was thawed in ice (matrigel is typically stored in -20°C conditions). After several minutes, 200μL of matrigel was added into 10 new cell culture dishes using a mechanical pipette. The matrigel filled culture dishes were incubated at 37°C for 30 minutes. While the dishes were incubating, the EC and EC+EV samples were collected from the incubator. As the EC and EC+EV were stuck to the bottom of the dishes, any solution in the samples was removed. PBS solution was pumped in and out of the cell culture dishes several times to clean the dish. 200μL of trypsin was added to each dish and incubated at 37°C for 2 minutes. 800μL of UCB mix was then added into each dish. 10 new eppendorfs were prepared. 5 of these eppendorfs were labels as "EC" while the remaining were labels as "EC+EV". 20μL of EC or EC+EV solution were moved into their respective eppendorfs. The remaining sample solutions were moved to 10 new centrifuge tubes, also with 5 tubes labels as "EC" and the remaining as "EC+EV". 4mL of UCB was added to each centrifuge tube and centrifuged under 1000rpm for 5 minutes. The supernatant was removed to 10 new cell pellets and labeled accordingly.

The 20μL of solution removed above was placed as a single drop on a hemocytometer. A slide cover was carefully placed on top of the single drop. The hemocytometer was placed under a microscope under x20 magnification.

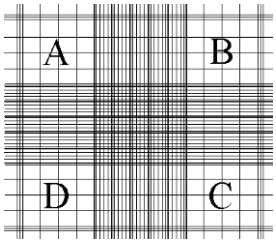

FIGURE 3: Hemocytometer Cell Count [20]

Cells visible in fields A, B, C, and D were counted. The hemocytometer was moved and fields counted again — therefore, a total of 8 fields was counted for each sample. This cell count procedure was repeated for all samples of "EC" and "EC+EV". Cell count was calculated using the formula: *cell count = (sum of cells counted)/number of boxes* $\times 10^4$. Final cell counts are recorded in *Calculations: Cell Count* below.

After cell counts were recorded, the 10 matrigel-filled dishes were removed from the incubator. Based on the data from *Calculations: In Vitro Volumes*, 360μL of "EC" solution was moved from the cell pellets into 5 matrigel-filled dishes. 1.14mL IMDM solution was also added to these 5 dishes and were labels as "EC". 1mL of "EC+EV" solution

and 0.5mL of IMDM solution was added into the 5 remaining matrigel filled dishes, and were accordingly labelled as "EC+EV". Photographs were taken under x20 magnification microscope for all 10 samples in 3 hour intervals (starting from 0 hours). Between each photograph, the 10 samples were incubated in 37°C environments.

CALCULATIONS

I. Volume for Angiogenesis Assay Samples

For 100% (or near complete) incorporation of EV to EC, there should be 0.2 nanogram of EV per $1x10^4$ EC. The volumes of EV will be adjusted to fit this ratio of EV to EC.

1. Each dish holds $3x10^5$ of EC (known)
2. EV Volume per $3x10^4$ of EC= 0.2/0.087=2.3mL
3. EV Volume/well= 2.3 × 30= 69mL

II. Cell Count

"EC"

Cell count = (sum of cells counted)/number of boxes x 10^4

= (34+29+50+61+48+49+41+59)/8 x 10^4 = 5 ×10^5cells/mL

"EC+EV"

Cell count = (17+22+15+17+14+23+16+20)/8 x 10^4

= 1.8 × 10^5cells/mL

III. In Vitro Volumes

As the number of cells in "EC" and "EC+EV" are different (as shown by the different cell counts), the volumes were adjusted so that "EC" samples have only $1.8x10^5$cells/mL.

Volume = $(1.8 \times 10^5)/(5 \times 10^5)$= 0.36mL = 360µL of "EC" per well.

EVALUATION OF ERRORS

As the volume for EV was adjusted for the best possible EC incoporation, nearly all EC should have incorporated with EV. However, as shown by the photos taken after incorporation, not all of the EC had EV incorporated in them. This could have been caused by slight differences in the volume of EV and EC (which could change the incorporation of cells by the hundreds). Luckily, although not all EC contained an EV, many of the EC did have EV incorporated in them, which is shown in *Incorporation Success*. The incorporation of EV could have been repeated to increase the number of successful incorporations; however, it would have been extremely expensive and time consuming to obtain more BCC samples from the hospital. Therefore, since there were still many EV which incorporated with EC, the investigation was continued.

Also, it must be considered that this investigation is conducted *in vitro*. While tube formation assays are widely regarded as reliable replicates of the body's microenvironment [23], the results obtained would be limited to an experimental scope of a petri dish which might not accurately represent how EV and EC interact in the body. Therefore, the results obtained are only predictions of the *likely* relationship between EC and EV in the body.

RESULTS

I. Incorporation Success

Before analyzing any results from the angiogenesis assay, the incorporation of EV with EC must show successful results. To determine this, a comparison of photos between EC and EC+EV must be compared to check whether or not the EV were incorporated in the EC. In methodology: Incorporation of EV to EC, the EV were stained with PKH26 and this stain was used to track EV. PKH26 is a fluorescent dye which stains the membranes by intercalating (inserting between DNA) with the EV's lipid bilayer [24]. The excitation maximum of PKH26 is 551 nm and emission maximum is 567nm. Under a red fluorescent light, the EV stained with PKH26 shine red and act as markers to indicate whether or not EV are found in the EC. PKH27 was used as it does not deteriorate even after several hours of EV incorporation. EC+EV were taken under both red fluorescent light (showing PKH26 stained EV) and white light (showing only EC), and these photos were combined to display where the PKH26 stained EV were located in the normal white light photo. The two photos were merged to observe whether or not the red EVs would be found in ECs in the white light photo.

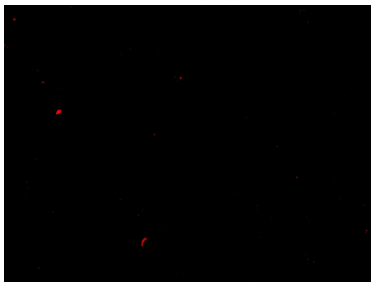

FIGURE 4: EC+EV under Red Fluorescent Light

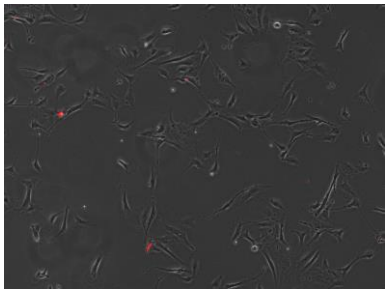

FIGURE 5: EC+EV Combined Photo with Red Fluorescent Light + White Light Photo

The red spots in Figure 4 justify the existence of PKH26 stained EV. After combined with the white light photo, Figure 5 exemplifies how the PKH26 stained EV are

physically spotted in the EC — thus showing that the incorporation of EV with EC was a success.

II. Tube Formation Photos

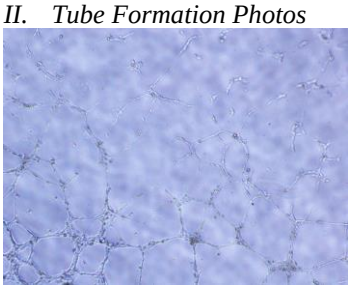

FIGURE 6: EC Tube Formation After 6 Hours

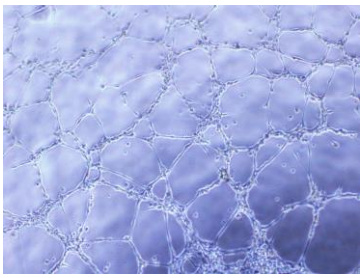

FIGURE 7: EC+EV Tube Formation After 6 Hours

Comparison between photos taken for both EC and EC+EV after 6 hours visibly show how tubes formed after EV incorporation created thicker and longer tubes with extensive networks. As these tube formations *in vitro* exemplify blood vessel formation in the body, the EV must have had an exchange of genetic material with EC to influence ECs to create thicker and longer blood vessels in our body. These blood vessels would therefore heighten the likelihood of metastasis as there are simply more pathways for the BCC to circulate throughout the body — supporting the hypothesis.

III. Tube Formation Length

Besides simple observation of tube formation, the average length of the tubes formed were calculated using ImageJ software [25].

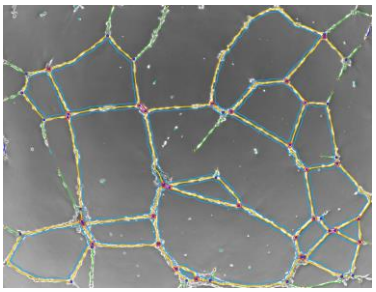

FIGURE 8: Example ImageJ Analysis of Tube Formation [25]

Using the photos taken during the 6 hour time frame, the computer software ImageJ performs an analysis on the skeleton of the tube formations, measuring the exact length of the tubes formed to the micrometer (shown in Figure 8). Using ImageJ and the photos of both "EC" and "EC+EV" after 0, 3, and 6 hours, the tube lengths shown below were calculated.

TABLE 1: Total Tube Lengths from ImageJ Scan

	EC (μm)	EC+EV (μm)
0 Hours	10526	16782
	11127	18790
	14248	14988
	16312	13459
	9569	15485
3 Hours	11969	13273
	9243	16625
	15742	14986
	15474	16789
	15496	15989
6 Hours	11183	16697
	11735	15691
	13176	15691
	11966	15502
	9822	13243

TABLE 2: Mean and Standard Deviations for Each Sample

	Mean (μm)	STDEV (μm)
EC (0 Hours)	12356.4	2822.07
EC+EV (0 Hours)	15900.8	2005.77
EC (3 Hours)	13584.8	2886.92
EC+EV (3 Hours)	15532.4	1447.82
EC (6 Hours)	13176.0	1221.51
EC+EV (6 Hours)	15364.8	1275.58

Comparisons between the mean for each hour show that the tubes formed after EV concentration was higher: a difference of 3,544.4 μm at 0 hours, 1,947.6 μm at 3 hours, and 2,188.8 μm at 6 hours. Using the standard deviations and means from the ImageJ measurements, the following graphs were made for each time interval.

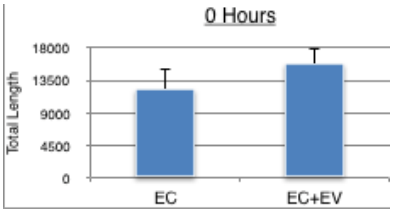

FIGURE 9: EC+EV Tube Total At 0 Hours

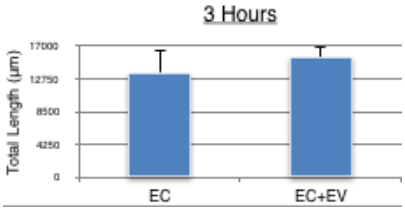

FIGURE 10: EC+EV Tube Total At 3 Hours

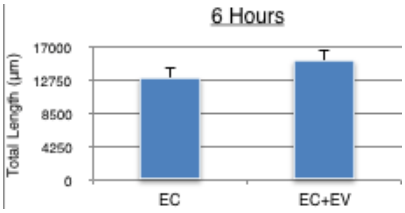

FIGURE 11: EC+EV Tube Total At 6 Hours

The graphs exemplify how the average tube length of the tubes formed were consistently higher for samples with added EV compared to control samples. Unfortunately, the error bars in both 0 hours and 3 hours were extremely large, indicating that there were no significant differences between the EC and EC+EV samples. However, the graph depicting tube formations after 6 hours show a smaller error bar — this graph will be used as the "model" tube formation result. After given enough time for tube formation to occur, the EC and EC+EV showed an obvious difference in tube length with little error compared to previous hours. Based on previous research on EV and EC (found in *Literature Review)* and the relative accuracy of past *in vitro* tube formations in mimicking angiogenic ability, it can be deduced that the presence of EV led to an increase in tube length. The recordings after 0 and 3 hours exemplify how total tube length at initial hours was not particularly influenced by EV incorporation when the error bars were taken into account. However, at 6 hours, the EV incorporation led to an increase in total tube length with relatively low error bars — indicating that EV incorporation alters tube formation behavior in *in vitro* environments and could possibly also alter angiogenic ability in the body. More tests and trials would need to be made to affirm the results of this investigation. The importance of EV in the role of angiogenesis, as shown by this investigation, would be vital in understanding how to control and prevent metastasis in cancerous cells.

CONCLUSION

In this investigation, the effects of BCC derived EV on angiogenesis was investigated through changes in EC activity, as EC play a vital role in the development of angiogenesis and in turn, influence metastasis. Comparisons between control samples of EC and variable samples of EC incorporated with EV exemplified that the BCC derived EV enhanced angiogenesis by creating lengthier and more complex tube formations in *in vitro* settings after 6 hours. These tubes mimic new blood vessel formation in the body, and an increase in tube length and formation after EV incorporation show that BCC derived EV are responsible in promoting angiogenesis and possibly metastasis of BCC. The results in this investigation show potential in further research on BCC-EV and endothelial cells — as shown in this investigation, the correlation between the two could prove to be essential in the limitation of metastasis in the future.

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